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Retinoid X Receptors Stimulate and 9-*cis* Retinoic Acid Inhibits 1,25-Dihydroxyvitamin D_3-Activated Expression of the Rat Osteocalcin Gene

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The vitamin D receptor (VDR) binds the vitamin D-responsive element (VDRE) as a heterodimer with an unidentified receptor auxiliary factor (RAF) present in mammalian cell nuclear extracts. VDR also interacts with the retinoid X receptors (RXRs), implying that RAF may be related to the RXRs. Here we demonstrate that highly purified HeLa cell RAF contained RXRβ immunoreactivity and that both activities copurified and precisely coeluted in high-resolution hydroxylapatite chromatography. Furthermore, an RXRβ-specific antibody disrupted VDR-RAF-VDRE complexes in mobility shift assays. These data strongly indicate that HeLa RAF is highly related to or is identical to RXRβ. Consequently, the effect of the 9-*cis* retinoic acid ligand for RXRs was examined in 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]-activated gene expression systems. Increasing concentrations of 9-*cis* retinoic acid (1 nM to 1 μM) markedly reduced 1,25$(OH)_2D_3$-dependent accumulation of osteocalcin mRNA in osteoblast-like ROS 17/2.8 cells. All-*trans* retinoic acid also interfered with vitamin D responsiveness, but it was consistently less potent than the 9-*cis* isomer. Transient transfection studies revealed that attenuation by 9-*cis* retinoic acid was at the transcriptional level and was mediated through interactions at the osteocalcin VDRE. Furthermore, overexpression of both RXRβ and RXRα augmented 1,25$(OH)_2D_3$ responsiveness in transient expression studies. Direct analysis of VDRE binding in mobility shift assays demonstrated that heteromeric interactions between VDR and RXR were enhanced by 1,25$(OH)_2D_3$ and were not affected appreciably by 9-*cis* retinoic acid, except that inhibition was observed at high retinoid concentrations. These data suggest a regulatory mechanism for osteocalcin gene expression that involves 1,25$(OH)_2D_3$-induced heterodimerization of VDR and unliganded RXR. 9-*cis* retinoic acid may attenuate 1,25$(OH)_2D_3$ responsiveness by diverting RXRs away from VDR-mediated transcription and towards other RXR-dependent transcriptional pathways.

The biological effects of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] are mediated, in part, through a soluble receptor protein termed the vitamin D receptor (VDR) (11). On the basis of sequence similarities, the VDR is classified as a member of the superfamily of nuclear receptors for steroid hormones, thyroid hormone, and retinoids (1, 29). Steroid receptors alter gene expression by binding to specific *cis*-acting DNA elements in the regulatory regions of hormone-responsive genes. For the glucocorticoid receptor (GR) and the estrogen receptor, the hormone-responsive elements are generally palindromic and the receptors bind as cooperative homodimers (19, 43, 45). The interaction of ligand, receptor, and response element culminates in a signal that eventually alters the rate of RNA polymerase II-catalyzed DNA transcription. The precise mechanism is not clear, but recent data suggest that enhancement is accomplished by receptor stabilization of the transcription preinitiation complex (15).

Vitamin D-responsive elements (VDREs) were identified in several genes that are induced by 1,25$(OH)_2D_3$, namely, the osteocalcin, osteopontin, and calbindin D_{9k} genes (4, 5, 14, 31–33, 42). Generally, VDREs consist of an imperfect direct repeat of the hexanucleotide sequence, GGGTGA, separated by a 3-nucleotide spacer. Similar direct repeat sequences serve as hormone-responsive elements for the thyroid and retinoid receptors (6, 8, 44). Accumulating evidence suggests that thyroid, retinoid, and vitamin D receptors bind to the direct repeat motifs not as homodimers but as heteromeric complexes in association with other receptors of the superfamily or with other unidentified nuclear factors (2, 9, 16, 17, 20, 22, 27, 46, 47). For example, DNA-binding studies demonstrated that highly purified VDR does not bind to the VDRE in vitro but requires an additional nuclear factor to facilitate high-affinity interaction with the element (22, 23, 36, 39). The nuclear accessory factor from HeLa cells forms a heterodimer with VDR, and this heteromeric interaction is facilitated by the 1,25$(OH)_2D_3$ ligand (39, 40).

Although the identity of this VDR auxiliary factor has not been established firmly, several lines of evidence allude to a potential candidate. Leid et al. purified a HeLa cell factor that facilitates retinoic acid receptor (RAR) and thyroid hormone receptor (TR) binding to their respective response elements (20). The factor was identified as the β isoform of retinoid X receptor (RXR), RXRβ. Several groups also demonstrated that recombinant RXRs heterodimerize with VDR and facilitate VDR-VDRE interactions in vitro (17, 46). These data suggest that the VDR auxiliary factor in HeLa cells may be RXR. In contrast, Yu et al. showed that RXRs activate VDR-mediated transcription only weakly in com-

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parison with their ability to enhance RAR- and TR-mediated events (46). Thus, the authors proposed that a novel, unidentified coregulator may be involved in VDR-dependent transcriptional activation.

A putative ligand for RXRs was identified as 9-*cis* retinoic acid on the basis of binding data and potency in transcriptional activation studies (12, 21). The 9-*cis* ligand synergistically activates heterodimer-mediated transcription by an RXR and several nuclear receptors, including TR (35) and the peroxisome proliferator receptor (18). There are limited data concerning 9-*cis* retinoic acid involvement in vitamin D-activated gene expression. Carlberg et al. presented evidence for RXR and 9-*cis* retinoic acid involvement in transcriptional activation of a restricted subclass of heterologous vitamin D-responsive constructs (3). Whether these data apply to natural promoter constructs or to expression of endogenous genes in appropriate target cells is not clear at present.

In the current study, our initial goal was to firmly establish the identity of the HeLa cell factor that facilitates VDR-VDRE interactions (termed receptor auxiliary factor [RAF]). We provide strong evidence that RAF is, or is highly related to, RXRβ. Secondly, we addressed the effects of 9-*cis* retinoic acid on vitamin D-mediated gene expression. Regulation of the vitamin D-responsive, bone-specific, rat osteocalcin gene (also called the bone GLA protein [BGP] gene) was examined at a variety of levels, including endogenous gene expression and transcriptional activity of natural osteocalcin promoter constructs. Our results demonstrate that 9-*cis* retinoic acid diminished vitamin D-activated transcription in an authentic vitamin D target cell line (ROS 17/2.8). In contrast, RXRs facilitated vitamin D-dependent expression, perhaps through heterodimer formation with VDR on the osteocalcin VDRE. These data suggest that 9-*cis* retinoic acid may attenuate VDR-activated osteocalcin transcription by diverting unliganded RXRs away from the transcriptionally active VDR-RXR heterodimer and towards other RXR-mediated pathways.

MATERIALS AND METHODS

Abbreviations. The following is a list of abbreviations used throughout this paper, in their order of appearance: vitamin D receptor, VDR; vitamin D-responsive element, VDRE; receptor auxiliary factor, RAF; retinoid X receptor, RXR; 1,25-dihydroxyvitamin D_3, $1,25(OH)_2D_3$; retinoic acid receptor, RAR; thyroid hormone receptor, TR; bone GLA protein or osteocalcin, BGP; baculovirus expression vector system, BEVS; Dulbecco's modified Eagle's medium, DMEM; glucocorticoid receptor, GR; glyceraldehyde 3-phosphate dehydrogenase, GAPDH.

Baculovirus-mediated expression of hVDR, RXRα, and RXRβ. The human vitamin D-receptor (hVDR) cDNA was incorporated into a BEVS as described previously (23). Human RXRα and murine RXRβ (mRXRβ) cDNAs were subcloned into the pVL1393 and pVL1392 polyhedrin transfer plasmids, respectively. Recombinant baculoviruses were isolated and plaque purified by standard procedures (41). Sf-9 cells were infected for 48 h with RXRα or RXRβ recombinant baculovirus. Whole-cell extracts were prepared by sonication of infected cell pellets in KETZD-0.3 (10 mM Tris-HCl [pH 7.8], 1 mM EDTA, 0.3 mM zinc acetate, 5 mM dithiothreitol, 0.3 M KCl), followed by centrifugation at 200,000 × *g* for 30 min. These extracts were snap-frozen and stored at −70°C.

Cell culture. ROS 17/2.8 cells were grown in DMEM-Ham's F12 (1:1) medium containing 5% newborn calf serum and 5% fetal bovine serum. CV-1 green monkey kidney cells were maintained in MEM with nonessential amino acids and 10% fetal bovine serum. HeLa S3 cells were grown as spinner cultures in MEM (Joklik modified) supplemented with 7% fetal bovine serum. All media contained 100 U of penicillin and 100 μg of streptomycin per ml.

Plasmids and transient transfection studies. The vitamin D-responsive $(CT4)^4$TK-GH growth hormone reporter plasmid (42) contains four copies of the rat osteocalcin VDRE upstream of the viral thymidine kinase promoter in the vector pTK-GH (37). BGP(−1000)-GH and BGP(−150)-GH contain 5′-flanking sequences of the rat BGP (osteocalcin) promoter of approximately 1,000 and 150 bp, respectively. These reporter constructs were termed prBGPGH and P2AGH in a previous publication (42). $CT4^1$-BGP(−150)-GH contains one copy of the osteocalcin VDRE adjacent to an osteocalcin promoter fragment in the construct BGP(−150)-GH. RSV-GH contains the Rous sarcoma virus promoter inserted upstream of the human growth hormone sequence in the plasmid p0GH. MMTV-CAT contains 1.4 kb of the hormone-inducible mouse mammary tumor virus long terminal repeat which drives expression of a chloramphenicol acetyltransferase (CAT) reporter gene. The pSG5hVDR expression plasmid was described elsewhere (13). Murine GR, human RXRα, and murine RXRβ cDNAs were inserted into the *Eco*RI site of pSG5 to generate pSG5mGR, pSG5h RXRα, and pSG5mRXRβ receptor expression plasmids, respectively.

ROS 17/2.8 cells and CV-1 cells were transfected by standard calcium phosphate coprecipitation procedures. A single DNA-calcium phosphate precipitate was prepared and applied to replicate plates for each treatment group. Carrier DNA (pTZ-18U) was added to bring the total DNA content to 20 μg per plate. Cells were incubated for 12 to 16 h with the precipitate. The precipitate was removed with two washes, and the cells were replenished with fresh media. All lipophilic ligands were dissolved in ethanol. The cells were treated with ligands or vehicle for 24 h, and the amount of secreted growth hormone was determined with a radioimmunoassay kit (Nichols Institute, San Juan Capistrano, Calif.). To examine glucocorticoid-dependent expression, ROS 17/2.8 cells were cotransfected with MMTV-CAT and pSG5mGR and then treated with ethanol vehicle, dexamethasone (10^{-6} M), and/or 9-*cis* retinoic acid (10^{-6} M). The cells were harvested after 24 h, cell lysates were prepared, and equivalent protein was assayed for CAT activity as described previously (7).

Northern (RNA) blot analysis. Subconfluent monolayers of ROS 17/2.8 cells were treated with ethanol or with 10^{-8} M $1,25(OH)_2D_3$ and various amounts of all-*trans* or 9-*cis* retinoic acid. The cells were harvested by trypsinization, and polyadenylated RNA was isolated with a commercially available kit (Invitrogen, San Diego, Calif.). A 2-μg amount of mRNA was separated on a 1.5% agarose-formaldehyde gel, transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.) by capillary action, and cross-linked by UV light. cDNA probes for hVDR, rat BGP, and rat GAPDH were labeled with [α-^{32}P]dCTP in random primer reactions with the Klenow fragment. The blots were hybridized with the labeled probe and washed as described previously (30).

HeLa RAF purification. Highly enriched preparations of HeLa cell RAF activity were obtained by standard chromatography, with buffer exchanges accomplished by overnight dialysis. Each column was monitored spectrophotometri-

cally for protein absorbance and by gel mobility shift analysis for RAF activity. All procedures were done at 4°C.

Frozen cell pellets representing approximately 5×10^{10} HeLa S3 cells (60 liters at 800,000 cells per ml) were used as a starting source. A whole-cell extract was prepared by the method of Manley et al. (26), except that ammonium sulfate was added at 0.24 g/ml in the final precipitation. The ammonium sulfate pellet was resuspended and dialyzed extensively against buffer D (20 mM HEPES, pH 7.9 [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]; 20% glycerol; 0.1 M KCl; 0.2 mM EDTA; and 1.0 mM dithiothreitol). The extract was applied to a Whatman P11 phosphocellulose column (12 by 2.5 cm) equilibrated in buffer D, and the column was washed until the protein absorbance returned to the baseline level. HeLa RAF activity was eluted with buffer D containing 0.5 M KCl (final concentration). The phosphocellulose pool was applied to a calf thymus DNA cellulose column (13.5 by 2.5 cm) equilibrated in KETZD-0.1. After the column had been given an extensive wash, RAF activity was eluted with KETZD-0.3. This material was concentrated by Amicon filtration with a YM-10 membrane and applied to a Sephacryl S-200 superfine column (60 by 2.6 cm) equilibrated in KETZD-0.4. The RAF activity was pooled and applied to a hydroxylapatite column (14-ml column volume) in 20 mM KH_2PO_4 (pH 7.2)–20% glycerol–1 mM dithiothreitol. RAF activity was eluted with a 60-ml linear phosphate gradient (0.02 to 0.4 M). RAF eluted at approximately 0.1 M KH_2PO_4 and was then applied to a heparin-agarose column (7 by 0.9 cm) in KETZD-0.1 containing 20% glycerol. Elution was with a 20-ml gradient of 0.1 to 0.6 M KCl. RAF activity was then subjected to a second hydroxylapatite step (column dimensions, 19 by 1 cm), and elution from this column was with a 50-ml gradient of 0.02 to 0.25 M KH_2PO_4. RAF activity from this final column was pooled, dialyzed into buffer D, snap-frozen, and stored at −70°C.

Antibody and Western blot (immunoblot) analyses. MOK 13.17 hybridoma cells (28) producing monoclonal antibody against mRXRβ were cultured in Iscove's modified Dulbecco's medium with 10% defined supplemented bovine calf serum (HyClone, Logan, Utah) and transferred to serum-free Iscove's modified Dulbecco's medium for antibody production. MOK 13.17 was purified from the hybridoma culture medium by ammonium sulfate precipitation, hydroxylapatite chromatography, and affinity chromatography on protein G-Sepharose. This preparation was over 95% pure as assessed by Coomassie blue staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The subunit molecular masses were approximately 55 and 24 kDa.

Fractions from the final hydroxylapatite column used in the HeLa RAF purification (described above) were separated on a 10% denaturing polyacrylamide gel and transferred to an Immobilon membrane, as described elsewhere (42). The membrane was processed by Western blotting protocols with highly purified MOK 13.17 antibody and iodinated sheep anti-mouse immunoglobulin G (28). Blots were incubated with immunoreagents in 1% BLOTTO, and washes were performed with Tris-buffered saline containing 0.1% Tween 20. Blots were used to expose X-ray film at −70°C.

Gel mobility shift analysis. Gel mobility shift analysis used procedures and conditions described previously (23). The VDRE probe was VDRE-WT from the rat osteocalcin gene, and specificity was examined with VDRE-MT, a mutated version of this probe (23). The glucocorticoid-responsive element (GRE) probe consists of the following sense-strand sequence: TCGACGGCGGTACACAGTGTTCTAGAC. The complementary strand was designed to yield *Sal*I and *Bam*HI overhangs after annealing. The retinoic acid-responsive element (RARE) probe contained the sense-strand sequence AGCTTAAGGGTTCACCGAAAGTTCACTCGC AT with *Hin*dIII overhangs. BEVS-hVDR, BEVS-RXRα, and BEVS-RXRβ were prepared as described above. BEVS-RXRα extract was a generous gift from R. Niles and T. Quick at Boston University Medical Center. GR-containing extracts were obtained by transfecting COS-7 cells with pSG5mGR and preparing nuclear extracts by the method of Shapiro et al. (38). Control COS-7 extracts were obtained by preparing similar extracts from cells transfected with the pSG5 plasmid lacking the cDNA insert. ROS 17/2.8 nuclear extracts were prepared as described elsewhere (38). Generally, proteins were preincubated for 20 min at 22°C followed by another 20-min incubation with ^{32}P-labeled response element probe. Unbound probe and protein-DNA complexes were separated by nondenaturing PAGE.

To examine ligand dependence in the mobility shift assay (see Fig. 9), the KCl concentration in the incubation solution was adjusted to 0.15 M. Protein extracts were treated individually with increasing amounts of ligand or vehicle for 30 min at 22°C. The liganded proteins were combined, preincubated with the mobility shift components for 20 min, and then incubated for an additional 20-min period with the labeled probe. These reaction mixtures were then separated as described above.

RESULTS

The VDR-dependent coreceptor (RAF) of HeLa cells is similar to RXRβ. Highly enriched preparations of human RAF activity were obtained following standard chromatography of HeLa cell extracts. Homogeneous hVDR was purified from a BEVS. Figure 1A illustrates the DNA-binding properties of purified hVDR and RAF. At these protein concentrations, baculovirus-expressed VDR and HeLa RAF did not interact appreciably with the VDRE of the rat osteocalcin gene in mobility shift assays (Fig. 1A, lanes 1 and 2). However, a protein-DNA complex was observed when the VDR and RAF preparations were coincubated with the labeled VDRE probe (Fig. 1A, lane 3). Both RXRα and RXRβ were similar to RAF in that these proteins alone did not bind to the VDRE (Fig. 1A, lanes 4 and 6), but coincubation of either protein with purified hVDR produced striking VDR-RXR-VDRE complexes (lanes 5 and 7). The ability of RAF and RXR to facilitate VDR-VDRE interactions was specific, as similar effects were not observed with an RARα-containing extract (Fig. 1A, lanes 8 and 9). Functional RARα was evident in this extract, since a specific RAR-DNA complex was observed when a labeled RARE probe was used (Fig. 1A, lanes 11 and 12). VDR had no effect on the RAR-RARE complex (Fig. 1A, lane 12). The intensity of the VDR-RXR complexes was greater than that of the RAF complex because of the limiting amounts of RAF in native HeLa extracts compared with the levels of RXR in the BEVS extracts. The RXRβ extracts occasionally generated two VDR-RXR complexes (Fig. 1A, lane 7). Western blot analysis showed that these early-generation extracts contained predominantly intact RXRβ (approximately 50 kDa) and a minor immunoreactive species of approximately 36 kDa (data not shown). Thus, heteromeric interactions may occur between VDR and either intact or truncated

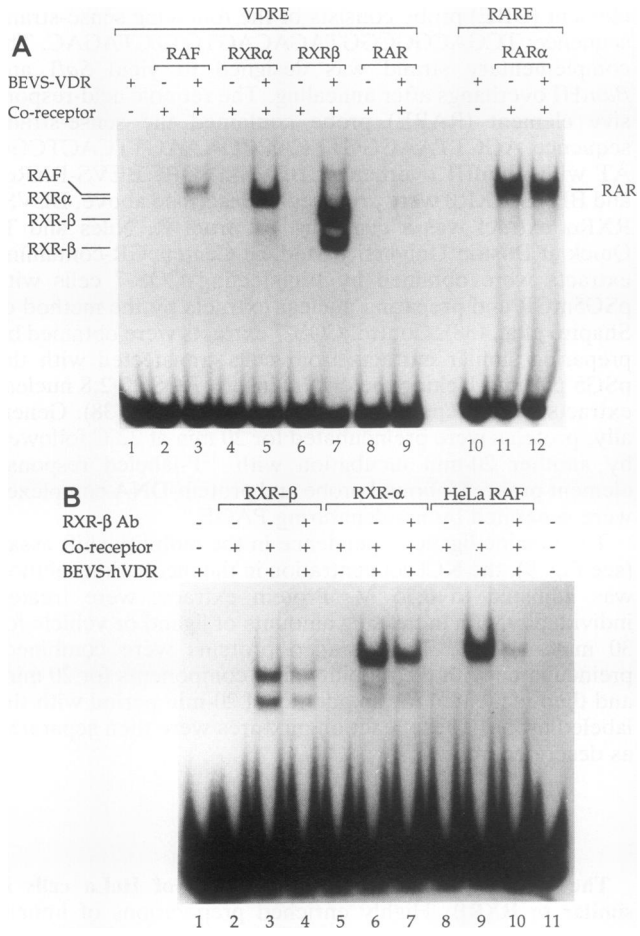

FIG. 1. (A) Mobility shift analysis of heteromeric interactions between purified BEVS-hVDR and HeLa RAF, human RXRα, and mRXRβ. HeLa cell RAF was highly enriched through the second hydroxylapatite step, as described in Materials and Methods (lanes 2 and 3). Sf-9 cell extracts containing BEVS-expressed RXRα (lanes 4 and 5), RXRβ (lanes 6 and 7), or RARα (lanes 8, 9, 11, and 12) were examined in the absence or presence of purified BEVS-expressed hVDR (10 ng of protein) and 0.5 ng of ^{32}P-labeled VDRE probe (lanes 1 to 9) or 0.5 ng of ^{32}P-labeled RARE-β probe (lanes 10 to 12). The protein-DNA complexes were resolved from free probe with a nondenaturing polyacrylamide gel and visualized by autoradiography of the dried gel. VDR-coreceptor complexes are indicated on the left. The RAR-RARE complex is indicated on the right. (B) Effect of monoclonal antibody against mRXRβ (RXR-β Ab) on the interaction of VDR with RXRβ, RXRα, and HeLa RAF. Conditions were similar to those described for panel A. Lanes 4, 7, and 10 show extracts that were preincubated with 10 ng (estimated) of purified MOK 13.17 antibody before probe addition.

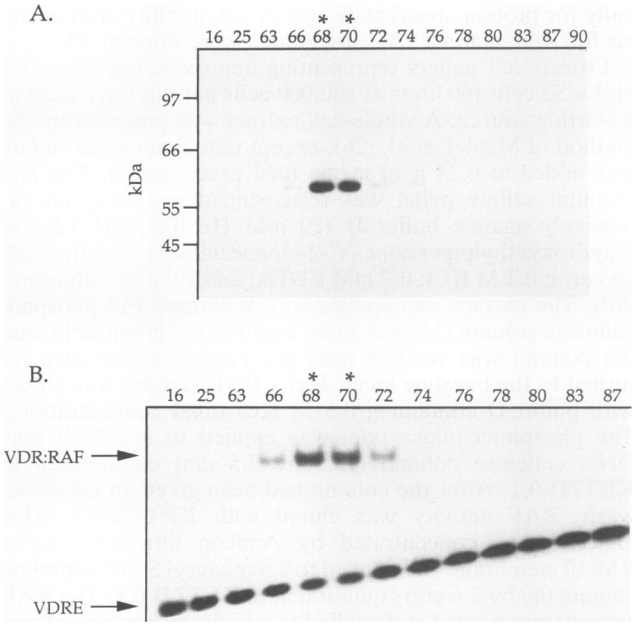

FIG. 2. (A) Elution profile of RXRβ immunoreactivity from hydroxylapatite, as assessed by Western blot analysis. HeLa RAF activity was purified as described in Materials and Methods. Individual fractions from the final hydroxylapatite column were fractionated on a 10% polyacrylamide denaturing gel and transferred electrophoretically to an Immobilon membrane. The membrane was processed successively with purified MOK 13.17 antibody against RXRβ and iodinated sheep anti-mouse immunoglobulin G. The membrane was used to expose X-ray film at −70°C for 24 h. (B) Elution profile of HeLa RAF from hydroxylapatite, as determined by a gel mobility shift assay. Identical fractions were also assayed for RAF activity in mobility shift analysis by combining the fraction with 10 ng of purified BEVS-hVDR and 0.5 ng of ^{32}P-labeled VDRE probe. For both panels, numbers along the top represent individual fractions. Note the exact coelution of RXRβ immunoreactivity and RAF activity from the final hydroxylapatite column (denoted by asterisks).

RXRβ. A similar effect was reported previously with BEVS-expressed mouse RXRβ (28).

Further evidence supporting the hypothesis that HeLa RAF may be similar to RXRs was obtained by mobility shift analysis with the MOK 13.17 monoclonal antibody raised against RXRβ (Fig. 1B). Preincubation of the MOK 13.17 antibody with RXRβ or RAF interfered with the formation of VDR-RXRβ-VDRE and VDR-RAF-VDRE complexes (Fig. 1B, lanes 4 and 10). This purified antibody was consistently less effective at disrupting VDR-RXRα-VDRE complex formation (Fig. 1B, lane 7). It is important to note that the MOK 13.17 antibody disrupts complexes here, while supershift effects were noted previously (28). This discrepancy is likely explained by the different systems examined. We studied VDR-RXR heterodimer binding to a VDRE, while Marks et al. (28) addressed RXR interaction alone on the RII response element.

Individual fractions from the final hydroxylapatite column used in the HeLa RAF purification were subjected to Western blot analysis with the RXRβ antibody. A 62,000-Da immunoreactive protein that eluted at approximately 0.1 M KH_2PO_4 and centered in fractions 68 and 70 was detected (Fig. 2A). The elution profile of RXRβ immunoreactivity was identical to the elution profile of HeLa RAF activity, as determined by gel mobility shift analysis (Fig. 2B). Both RXRβ immunoreactivity and highly purified HeLa RAF activity comigrated precisely in this system and were resolved from contaminating proteins whose elution was centered at fraction 74 (data not shown).

The VDR-dependent coreceptor (RAF) of ROS 17/2.8 cells is also similar to RXRβ. Further evidence for the functional relevance of RXRβ as a coreceptor in VDR-VDRE interactions was obtained by examining endogenous receptor proteins in nuclear extracts obtained from the vitamin D target cell line, ROS 17/2.8. This osteosarcoma cell line expresses functional VDR and responds to $1,25(OH)_2D_3$ by enhancing

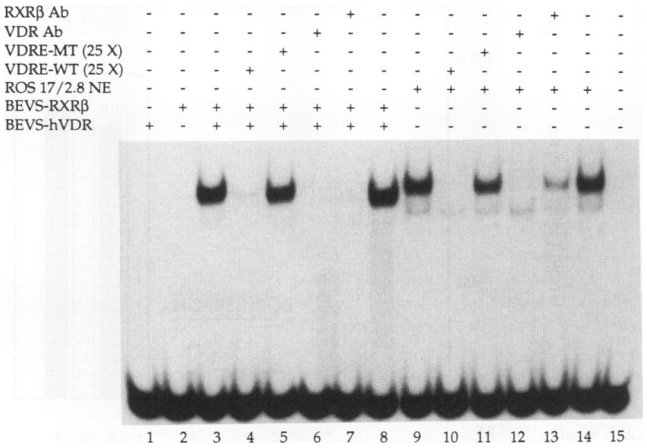

FIG. 3. RXRβ antibody (RXRβ Ab) disrupts binding of native ROS 17/2.8 VDR-RAF heterodimers to the rat osteocalcin VDRE. Conditions are similar to those described in the legend to Fig. 1, except that a separate RXRβ extract containing predominantly intact RXRβ was used in lanes 2 to 8. Thus, only the higher of the two complexes observed in Fig. 1 is apparent here. Lanes 9 to 14 utilize a nuclear extract (NE) prepared from ROS 17/2.8 cells. Protein-DNA complexes were examined in the presence of a 25-fold excess of unlabeled VDRE probe (VDRE-WT) (lanes 4 and 10), a 25-fold excess of unlabeled mutated VDRE (VDRE-MT) (lanes 5 and 11), or monoclonal antibody against VDR (VDR Ab) (lanes 6 and 12) or against RXRβ (lanes 7 and 13).

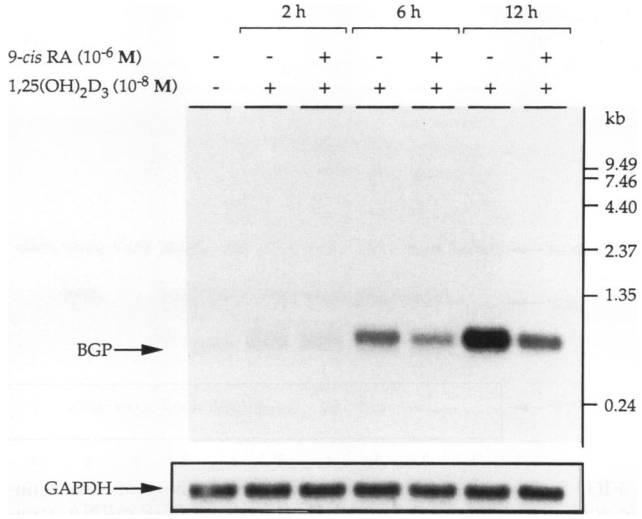

FIG. 4. Northern blot analysis of steady-state BGP mRNA levels following treatment of ROS 17/2.8 cells with $1,25(OH)_2D_3$ and 9-*cis* retinoic acid. Log-phase cultures of ROS 17/2.8 cells were treated with ethanol vehicle, with 10 nM $1,25(OH)_2D_3$, or with a combination of 10 nM $1,25(OH)_2D_3$ and 1 μM 9-*cis* retinoic acid for 2, 6, or 12 h. Polyadenylated RNA was isolated, and a 2-μg amount was separated on a 1.5% agarose-formaldehyde gel. The RNA was transferred to a Nytran membrane, and BGP mRNA was identified by standard Northern blotting procedures. The 0.5-kb rat BGP transcript is indicated on the left. The control lane (−) contains cells treated for 6 h with ethanol vehicle. The blot was stripped of radiolabeled probe in 50% formamide–6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) at 65°C for 3 h and then hybridized with the labeled probe for human GAPDH (inset). The GAPDH transcript is not regulated by $1,25(OH)_2D_3$ and serves as an internal control for poly(A)$^+$ mRNA loading. 9-*cis* RA, 9-*cis* retinoic acid.

transcription of the osteocalcin gene. As illustrated in Fig. 3, a protein-DNA complex was observed with the crude nuclear extract and the labeled VDRE probe (lanes 9 and 14). This complex was eliminated by unlabeled probe (Fig. 3, lane 10) and was not affected by a mutated version of the probe (lane 11). Moreover, monoclonal antibodies raised against VDR (Fig. 3, lane 12) or RXRβ (lane 13) interfered with complex formation, illustrating that both the VDR and RXRβ coreceptors were present in the complex. The recombinant hVDR and mRXRβ generated a complex with properties similar to those of the ROS 17/2.8 nuclear extract (Fig. 3, lanes 1 to 8). This particular RXRβ extract contains predominantly intact coreceptor as assessed by Western blot (data not shown) and therefore yields mainly the higher of the two complexes seen in Fig. 1.

Effects of 9-*cis* retinoic acid on vitamin D-mediated gene expression. On the basis of the similarities between RAF and RXR observed in the mobility shift studies, we examined the effects of 9-*cis* retinoic acid on vitamin D-mediated gene expression. First, we examined retinoid effects on $1,25(OH)_2D_3$-activated expression of endogenous genes. ROS 17/2.8 cells were cultured in the absence and presence of $1,25(OH)_2D_3$ and 9-*cis* retinoic acid for various times, and polyadenylated RNA was isolated and subjected to Northern blot analysis (Fig. 4). Following 6- and 12-h treatments with 10 nM $1,25(OH)_2D_3$, fourfold and sevenfold inductions in steady-state BGP mRNA levels were observed, respectively. This $1,25(OH)_2D_3$-dependent accumulation was attenuated (>40% reduction) when the cells were incubated with a combination of 10 nM $1,25(OH)_2D_3$ and 1 μM 9-*cis* retinoic acid. No differences in the steady-state mRNA levels for GAPDH were noted in any of the treatment groups (Fig. 4).

Figure 5 shows that this effect was dependent on the concentration of retinoid. Scanning densitometry revealed substantial attenuation at 10 nM 9-*cis* retinoic acid (a 13% reduction) and a maximal effect (a 60% reduction) at 1 μM (Fig. 5, lanes 3 to 7). A diminution of vitamin D-dependent accumulation of BGP mRNA was elicited by all-*trans* retinoic acid as well (Fig. 5, lane 9), but the effect was reproducibly less evident with this isomer (compare lanes 6 and 9). The 9-*cis* and all-*trans* retinoids alone had no effect on steady-state BGP mRNA levels (Fig. 5, lanes 8 and 10). Interestingly, VDR mRNA levels (Fig. 5) were increased by treatment with $1,25(OH)_2D_3$ and retinoids, either alone or in combination. Since retinoids increased VDR mRNA levels and since others have demonstrated previously that retinoids also increase VDR protein levels in ROS 17/2.8 cells (34), it is likely that the suppressive action of retinoids was not due to diminished levels of functional VDR in these cells.

The decrease in vitamin D-activated gene expression elicited by 9-*cis* retinoic acid was also observed in transient transfection of ROS 17/2.8 cells with a vitamin D-responsive reporter construct (Fig. 6). BGP(−1000)-GH contains approximately 1,000 bp of 5′-flanking DNA from the rat osteocalcin gene linked to a human growth hormone reporter sequence. Transfection of this construct into ROS 17/2.8 cells, followed by treatment with 10 nM $1,25(OH)_2D_3$, resulted in an eightfold increase in growth hormone reporter expression (Fig. 6A). This $1,25(OH)_2D_3$-dependent increase was diminished 56% by cotreatment with 1 μM 9-*cis* retinoic acid. The all-*trans* isomer produced a similar response, but again, it was less effective (causing a 26% reduction) than

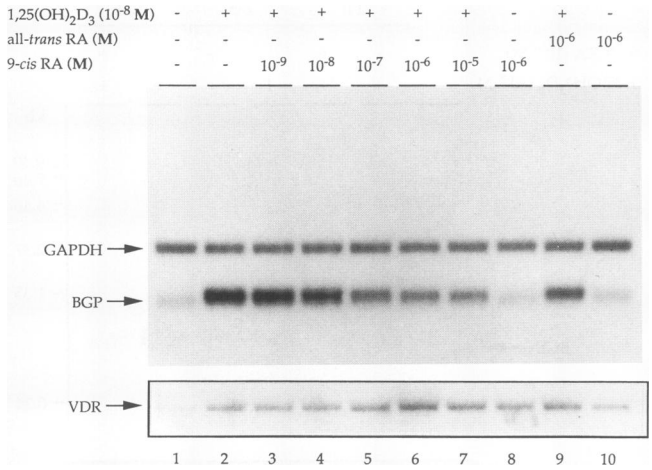

FIG. 5. Dose dependence and retinoid selectivity of 9-*cis* retinoic acid in suppressing $1,25(OH)_2D_3$-stimulated BGP mRNA accumulation. Subconfluent monolayers of ROS 17/2.8 cells were treated with 10 nM $1,25(OH)_2D_3$ and increasing amounts of 9-*cis* retinoic acid for 12 h. Polyadenylated RNA was isolated from each group, and 2 μg was subjected to Northern blot analysis. GAPDH and BGP probes were hybridized at the same time. The blot was then stripped and reprobed with labeled hVDR cDNA (inset). 9-*cis* RA, 9-*cis* retinoic acid; all-*trans* RA, all-*trans* retinoic acid.

9-*cis* retinoic acid in this system. This effect of the vitamin D or A ligands was not observed with the BGP(−150)-GH basal promoter construct or with the Rous sarcoma virus promoter (Fig. 6A). Importantly, a single copy of the rat osteocalcin VDRE engineered 5′ to the basal osteocalcin promoter [CT4[1]-BGP(−150)-GH] restored $1,25(OH)_2D_3$ responsiveness and also inhibition by retinoic acid (Fig. 6B). There was modest inhibition of CT4[1]-BGP(−150)-GH expression by 9-*cis* retinoic acid alone. This was not apparent with the BGP(−150)-GH control vector or with the other vectors used in this study. This may represent suppression of a low level of vitamin D-mediated expression in ethanol-treated controls because of residual $1,25(OH)_2D_3$ in the serum of the culture medium. The observation that the basal expression of the CT4[1]-BGP(−150)-GH construct is higher than that of the BGP(−150)-GH control supports this possibility.

To examine the specificity of the suppressive effect with respect to other steroid hormone-activated systems, we examined the influence of 9-*cis* retinoic acid on glucocorticoid-mediated reporter expression in the ROS 17/2.8 cell line (Fig. 7). ROS 17/2.8 cells, cotransfected with MMTV-CAT and a GR expression vector, expressed similar levels of glucocorticoid-induced CAT activity when treated with dexamethasone or with a combination of dexamethasone and 9-*cis* retinoic acid. Therefore, the influence of retinoic acid on vitamin D-mediated gene expression was not the result of a generalized decrease in hormone-induced gene expression.

Effects of RXRs and 9-*cis* retinoic acid on vitamin D-responsive heterologous promoter constructs. To obtain additional evidence that the retinoid effects were mediated through the VDRE of the rat osteocalcin promoter, studies were conducted with (CT4)[4]TK-GH. This reporter vector contains multiple copies of the rat osteocalcin VDRE linked to the herpes simplex virus thymidine kinase promoter, which drives expression of the growth hormone reporter gene.

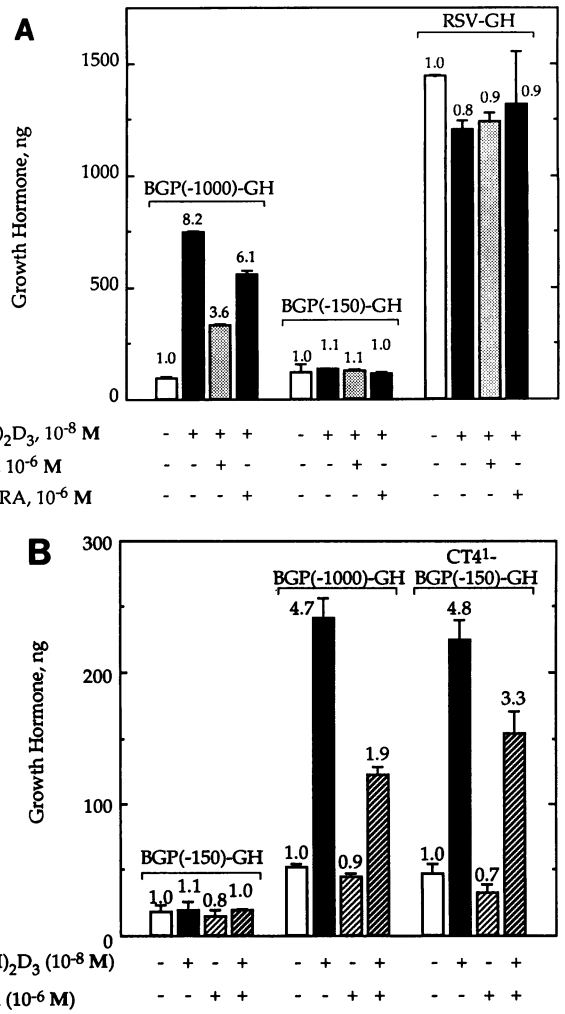

FIG. 6. (A) Selective attenuation of vitamin D-mediated BGP promoter activity by 9-*cis* retinoic acid in ROS 17/2.8 cells. ROS 17/2.8 cells were plated at 10^6 cells per 100-mm plate. Each plate was transfected 24 h later by calcium phosphate precipitation with pTZ-18U carrier DNA (to 20 μg of total DNA per plate) and either 10 μg of BGP(−1000)-GH, 10 μg of BGP(−150)-GH, or 1 μg of RSV-GH. Duplicate plates were treated with ethanol vehicle (open bars), with 10 nM $1,25(OH)_2D_3$ (solid bars), with a combination of 10 nM $1,25(OH)_2D_3$ and 1 μM 9-*cis* retinoic acid (light stippled bars), or with a combination of 10 nM $1,25(OH)_2D_3$ and 1 μM all-*trans* retinoic acid (dark stippled bars). 24 h later, secreted growth hormone was quantitated by radioimmunoassay. (B) $1,25(OH)_2D_3$ and 9-*cis* retinoic acid effects are both mediated through the VDRE. This separate, representative experiment used conditions similar to those used for the experiment described above and also examined the CT4[1]-BGP(−150)-GH reporter vector. Results are presented as means and standard deviations (error bars). For each treatment group, $n = 3$. 9-*cis* RA, 9-*cis* retinoic acid; all-*trans* RA, all-*trans* retinoic acid. The number above each bar represents the fold induction in comparison with ethanol-treated controls.

Therefore, this heterologous construct lacks all osteocalcin promoter sequences except the VDREs. COS-7 cells and CV-1 cells were used to examine the effects of VDR and RXR expression plasmids, since both lines express low levels of endogenous steroid receptors.

As illustrated in Fig. 8A, COS-7 cells cotransfected with the (CT4)[4]TK-GH reporter and the SG5hVDR expression

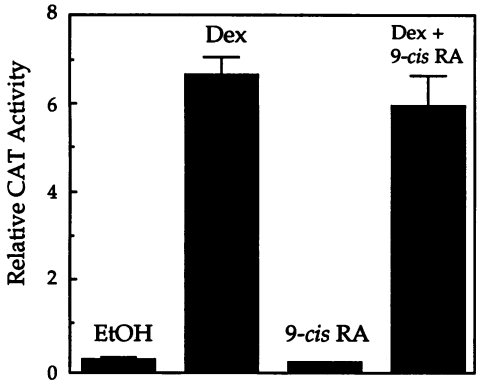

FIG. 7. 9-*cis* retinoic acid does not affect glucocorticoid-dependent expression of MMTV-CAT in ROS 17/2.8. ROS 17/2.8 cells were plated and transfected as described in the legend to Fig. 6. Each plate received 2 μg of MMTV-CAT, 100 ng of pSG5mGR expression plasmid, and 17.9 μg of pTZ-18U carrier DNA. Duplicate plates were treated with ethanol vehicle, 1 μM dexamethasone, 1 μM 9-*cis* retinoic acid, or a combination of dexamethasone and 9-*cis* retinoic acid. The CAT assay autoradiogram was quantitated with a Betagen beta scope blot analyzer. Results are presented as means and standard deviations (error bars). EtOH, ethanol vehicle; Dex, dexamethasone; 9-*cis* RA, 9-*cis* retinoic acid.

plasmid exhibited a dramatic 36-fold induction in growth hormone expression in the presence of 10 nM $1,25(OH)_2D_3$. As in the previous experiments, this effect was blunted approximately 73% by coincubation with $1,25(OH)_2D_3$ and 1 μM 9-*cis* retinoic acid. Coexpression of RXRα or RXRβ with VDR generated a further increase in vitamin D responsiveness compared with transfections with VDR expression plasmid alone (a 58-fold induction compared with a 36-fold induction). Again, the addition of the ligand for RXR generated a marked suppression of vitamin D-activated reporter expression. Vitamin D and/or A metabolites had no effect on basal expression in cells transfected with the pTK-GH enhancerless control vector (data not shown). Additional studies with CV-1 cells (Fig. 8B) showed that both RXRα and RXRβ cooperated with the VDR to augment $1,25(OH)_2D_3$-induced expression. Moreover, the inhibitory effects of 9-*cis* retinoic acid were also apparent but much less pronounced in the CV-1 cell line. These studies provide further evidence that both the stimulation by RXR and the attenuation by 9-*cis* retinoic acid of $1,25(OH)_2D_3$-activated gene expression were transcriptional events mediated through interactions at the VDRE.

$1,25(OH)_2D_3$ enhances binding of VDR-RXR heterodimers to the VDRE. Finally, we examined the individual and combined effects of $1,25(OH)_2D_3$ and 9-*cis* retinoic acid on binding of VDR-RXR heterodimers to the VDRE in vitro. Mobility shift analysis revealed a dramatic increase in heteromeric complex formation of VDR-RXR-VDRE when 10 nM $1,25(OH)_2D_3$ was included in the assay (Fig. 9, lanes 5 and 6). Increasing concentrations of 9-*cis* retinoic acid had little effect on VDR-RXR heterodimer binding except when present at 10^{-5} M. At this concentration, it was somewhat inhibitory (Fig. 9, lanes 4 and 9). Although this inhibition occurred at relatively high concentrations of 9-*cis* retinoic acid, elements of specificity were apparent in that similar concentrations of 9-*cis* retinoic acid did not alter GR binding to a glucocorticoid-responsive element (lanes 11 and 12).

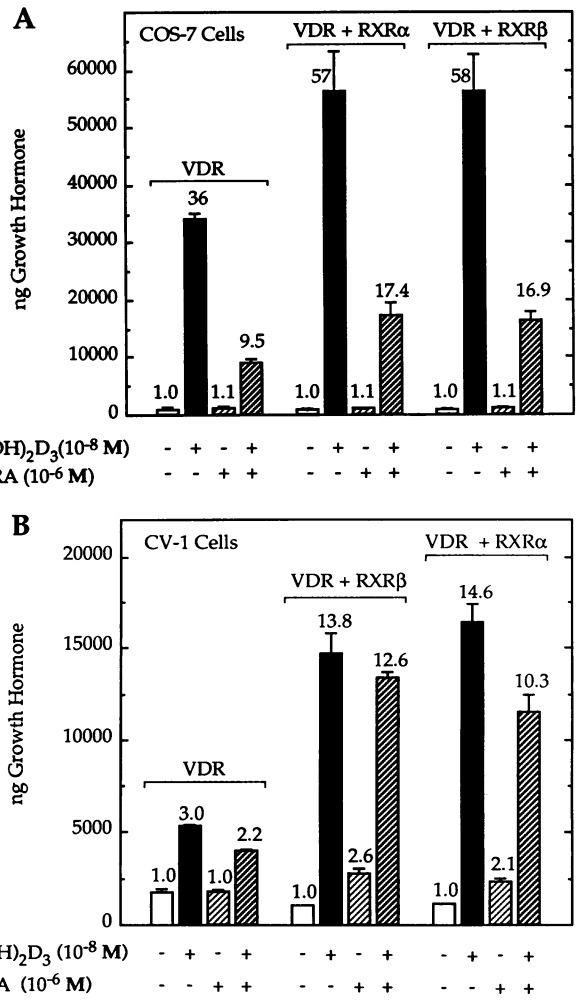

FIG. 8. Attenuation of vitamin D-activated gene expression by 9-*cis* retinoic acid is mediated through the VDRE. (A) COS-7 cells were plated at 400,000 cells per 60-mm plate and transfected 6 h later as described in the legend to Fig. 6. Each plate received 7.5 μg of $(CT4)^4$TK-GH, 2.5 μg of pSG5hVDR expression plasmid, 0.5 μg of either pSG5 (left group), pSG5hRXRα (middle group), or pSG5mRXRβ expression plasmid (right group), and pTZ-18U to 20 μg of total DNA. Triplicate plates were treated with ligands as indicated, and growth hormone was quantitated 24 h later. (B) CV-1 cells were plated and transfected as described for panel A. Each plate received 3.5 μg of $(CT4)^4$TK-GH; 2.0 μg of pSG5hVDR expression plasmid; 100 ng of either pSG5 (left group), pSG5RXRβ (middle group), or pSG5RXRα (right group); and pTZ-18U to 20 μg of total DNA per plate. Results are presented as means and standard deviations (error bars). The number above each bar represents the fold induction in comparison with ethanol-treated controls.

DISCUSSION

An RAF in mammalian cell nuclear extracts heterodimerizes with VDR and is essential for high-affinity VDR-VDRE interactions (22, 23, 39, 40). Recombinant RXRs also interact with VDR (17, 46). In the present study, we have isolated native HeLa cell RAF activity and demonstrated that it is highly related or identical to the β isoform of RXR. First, HeLa RAF activity and RXRβ immunoreactivity copurified through a seven-step enrichment protocol and both activities demonstrated precise coelution from the final hydroxylapatite column. The 62,000-Da immunoreactive species and the

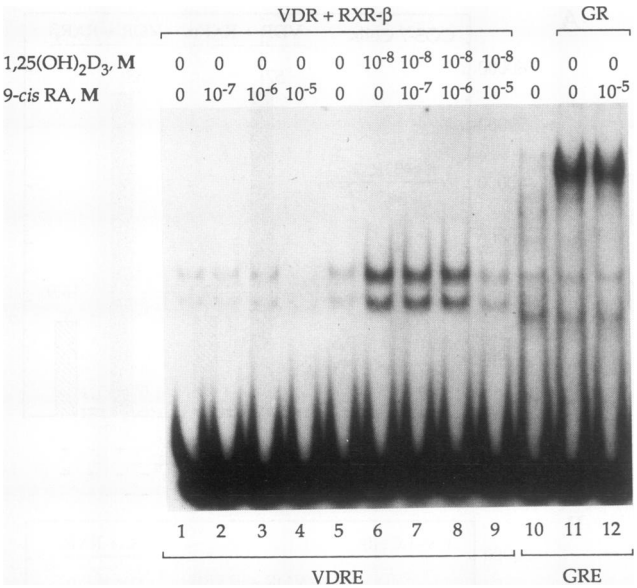

FIG. 9. Mobility shift analysis showing that $1,25(OH)_2D_3$ stimulates and 9-*cis* retinoic acid inhibits VDR-RXR-VDRE complex formation. Ligand-free BEVS-hVDR was obtained from infected Sf-9 cells (lanes 1 to 9). GR preparations were obtained as nuclear extracts from COS-7 cells transfected with pSG5mGR (lanes 11 and 12). Lane 10 contains a similarly prepared nuclear extract from COS-7 cells transfected with pSG5 as a control. Individual receptor preparations were incubated with ethanol vehicle, $1,25(OH)_2D_3$, or 9-*cis* retinoic acid for 30 min at 22°C. The extracts were mixed and then examined in the mobility shift assay. The final concentrations of ligand in the mobility shift assay are indicated. 9-*cis* RA, 9-*cis* retinoic acid.

HeLa RAF activity were eluted with approximately 0.1 M phosphate, agreeing well with the molecular mass and elution characteristics of human RXRβ characterized previously (20). Secondly, a homogeneous RXRβ-specific antibody disrupted both VDR-RXRβ and VDR-RAF heteromeric binding to the VDRE, but it did not affect VDR-RXRα heterodimers to the same extent. This antibody also inhibited VDRE binding of native VDR-RAF heterodimers obtained from the vitamin D-responsive cell line, ROS 17/2.8. Finally, cotransfection of CV-1 and COS-7 cells with VDR and RXR expression plasmids provided functional evidence that RXRs participate in $1,25(OH)_2D_3$-stimulated, VDR-dependent transcription. Consequently, these data strongly suggest that HeLa RAF is RXR, and they illuminate the functional relevance of RXRs in vitamin D action.

One caveat of note is that the RAF (from HeLa and ROS 17/2.8 cells) and the BEVS-expressed mRXRβ complexes do not migrate similarly in mobility shift assays (Fig. 1 and 3). We and others (20) estimate by SDS-PAGE that the molecular mass of human RXRβ is 62,000 to 64,000 Da and that of mRXRβ generated in the BEVS is 51,000 Da (28). Thus, it is unlikely that these RXRβ isoforms would exhibit similar migration rates in the mobility shift assay. Moreover, initiator methionines inferred from the cDNA sequences of the human RXRβ (20), rat RXRβ (46), and mRXRβ (10, 25) isoforms confirm this mass difference and further suggest the following size order: human RXRβ > rat RXRβ > mRXRβ. The migration of the various RAF complexes in the mobility shift assay reflects this difference in molecular mass (i.e., HeLa RAF > ROS 17/2.8 RAF > BEVS-mRXRβ). The human and rat variants of VDR also differ, but to a lesser extent (24). Therefore, migration rates of the VDR-RXR heteromeric complexes in the mobility shift studies may be due to species-related size differences of RXRβ.

Although the evidence clearly points to RXRβ as RAF in HeLa cells, and possibly in ROS 17/2.8 cells as well, it is also true that other isoforms of RXR (namely, RXRα) promote VDR-VDRE interaction and augment VDR-mediated transcription. That the β isoform of RXR was identified in HeLa cells may simply reflect the relative abundance of RXRβ compared with the other RXR isoforms in HeLa cells. For example, the MOK 13.17 antibody only partially disrupted (approximately 80%) the VDR-RAF heteromeric complex in crude nuclear extracts obtained from ROS 17/2.8 cells. Increasing the antibody/antigen ratio had no further effect on the residual complex (data not shown). This would suggest that, in addition to the predominant role of RXRβ, other factors in the ROS 17/2.8 nuclear extract participate in VDR-VDRE interactions. This factor may be another isoform of RXR or possibly a highly related protein. Thus, our studies do not rule out the possibility suggested by Yu et al. that other unidentified, RXR-related factors may function as coreceptors in VDR-dependent transcriptional responses (46). It is conceivable that VDR coreceptors differ in selected vitamin D target cells, in the regulation of distinct vitamin D-controlled genes, or at various stages of target cell differentiation.

Combinatorial diversity refers to the myriad of coreceptor combinations that could each yield functionally distinct transcriptional activators. With respect to the steroid receptor superfamily, ligands for the individual receptors add another level of regulatory complexity. For example, ligands for each coreceptor might elicit synergistic or opposing effects on the ultimate transcriptional activity of the heterodimer. Kliewer et al. showed recently that the 9-*cis* retinoic acid ligand acts synergistically with the peroxisome-proliferating agent, clofibrate, to enhance expression of acyl CoA oxidase promoter constructs through heterodimer binding of RXR and peroxisome proliferator-activated protein to the peroxisome proliferator response element (18). In contrast to synergistic activation, we demonstrate that retinoids counteract $1,25(OH)_2D_3$-dependent expression of the rat osteocalcin gene. This inhibitory effect was evident at several levels of osteocalcin expression in vitamin D-responsive target cells and in other cell types. In the osteoblast-like target cell line, ROS 17/2.8, activation of the endogenous osteocalcin gene by vitamin D (monitored indirectly by Northern blot analysis) was rapidly attenuated by 9-*cis* retinoic acid (Fig. 4). This effect was reproduced by direct analysis of the natural osteocalcin promoter activity in transient expression studies with ROS 17/2.8 cells (Fig. 6). The 9-*cis* isomer was more potent than all-*trans* retinoic acid in both systems, thus lending support to the involvement of RXR in vitamin D-activated osteocalcin gene expression. Moreover, our evidence suggests that the attenuation of $1,25(OH)_2D_3$-induced transcription by retinoic acid is mediated through the VDRE of the rat osteocalcin gene. A single copy of the VDRE adjacent to the basal osteocalcin promoter restored both $1,25(OH)_2D_3$ inducibility and inhibition by retinoic acid in this system. Furthermore, the VDRE conferred both these activities on heterologous thymidine kinase promoter constructs in COS-7 cells. Inhibition by retinoic acid was less profound in CV-1 cells, suggesting that this may not be a general mechanism for all vitamin D-responsive genes or for all cell types. For example, those cells that metabolize retinoids differently or that express different

levels or isoforms of the cellular binding proteins for retinoids may exhibit different capacities to respond to vitamins A and D in this coordinated fashion. Importantly, in an authentic vitamin D-responsive osteoblast-like cell line, there is a profound inhibition of vitamin D-activated osteocalcin gene expression by retinoic acid.

Our data conflict to some extent with data obtained in recent studies by Carlberg et al. (3), who showed that selected synthetic VDREs responded differentially to the effects of RXR and 9-*cis* retinoic acid. Two distinct pathways for vitamin D-activated gene expression were proposed: (i) an RXR- and 9-*cis* retinoic acid-dependent pathway and (ii) an RXR- and 9-*cis* retinoic acid-independent pathway mediated through VDR homodimerization. In their study (3), VDR did not heterodimerize with RXR on the human osteocalcin VDRE. In contrast, we and others (46) have demonstrated that VDR readily forms heteromeric complexes with both RXRα and RXRβ on the rat and human osteocalcin VDREs. Using nanogram quantities of well-characterized BEVS-expressed receptors, we observe highly specific VDRE complexes only when VDR and RXR are present together in the incubation mixture. The heteromeric nature of the complex is also indicated by the ability of individual monoclonal antibodies against each coreceptor to interfere with complex formation (Fig. 3). In addition, Carlberg et al. reported no effect of RXR on VDR-activated transcription of osteocalcin VDRE reporter constructs (3). However, our data and the original observation of Yu et al. (46) together show that both RXRα and RXRβ augment VDR-dependent expression of heterologous constructs containing the osteocalcin VDRE (Fig. 8). A possible explanation for these discrepancies is that the human osteocalcin VDRE utilized by Carlberg et al. contains additional sequence outside the minimal VDRE used here. Moreover, a point mutation was introduced such that the TGGTGA sequence at positions −511 to −516 in the human osteocalcin promoter (31, 33), was altered to GGGTGA in their studies. Alternatively, our reporter construct contains tandem copies of the minimal VDRE which could create adventitious activation elements. However, Yu et al. used a single copy of the VDRE in a similar vector to show RXR participation in VDR-mediated transactivation (46). Finally, Carlberg et al. used *Drosophila* SL-3 and MCF 7 cell lines as recipients. Thus, it will be intriguing to determine whether responses to RXR and 9-*cis* retinoic acid as they relate to VDR-dependent mechanisms are cell-type selective. This would add another level of regulatory complexity to the distinct vitamin D-responsive pathways proposed in that provocative report.

Since activation by $1,25(OH)_2D_3$ and inhibition by 9-*cis* retinoic acid were mediated through the osteocalcin VDRE in the present study, we reasoned that the retinoid might interfere directly with the formation of functional VDR-RXR heterodimers. Therefore, the influence of the ligands on VDR-RXR interactions with the VDRE was examined. $1,25(OH)_2D_3$ increased binding of VDR and unliganded RXR to the VDRE. Such ligand-enhanced binding of VDR to the VDRE was reported previously for VDR-RAF heterodimers (22, 39), and we extend that observation to include VDR-RXR complexes as well. Thus, RAF and RXR share another functional property in that both activities favor heterodimerization with the liganded form of VDR. Under these conditions, 9-*cis* retinoic acid did not alter VDR-RXR-VDRE interactions appreciably, unless rather large amounts of retinoic acid were added. Only then did we observe modest, yet specific, inhibition of VDR-RXR complexes. Therefore, we conclude that under these in vitro conditions, liganded VDR interacts equally well with either unliganded RXR or liganded RXR. Retinoic acid does not interfere directly with VDR-RXR heterodimer formation, but it may do so indirectly. For example, it is possible that $1,25(OH)_2D_3$ binds to the VDR and promotes VDR interaction with unliganded RXRs or with RXR-related coreceptors. The heterodimer of holoVDR-apoRXR may be the functional activator in certain vitamin D-mediated systems. The 9-*cis* retinoic acid ligand may divert RXRs away from VDR-dependent pathways and towards other RXR-mediated events in which holoRXR is the functional coreceptor. In this regard, Zhang et al. (48) demonstrated that 9-*cis* retinoic acid induced homodimer formation of RXR, and they hypothesized that the equilibrium between homodimers and heterodimers may control distinct ligand-responsive pathways. For VDR and osteocalcin gene expression, 9-*cis* retinoic acid might shift the RXR equilibrium away from vitamin D-activated expression of bone matrix proteins and towards expression of vitamin A-dependent gene networks. This may provide a novel means to deregulate or turn off hormone-activated gene expression in complex processes such as cellular specialization or differentiation, when entire subsets of genes are simultaneously activated and repressed.

Finally, a mechanism for vitamin D-dependent gene expression involving liganded VDR and unliganded RXR or RXR-related coreceptors may help explain the lack of obvious connections between the actions of vitamins D and A in vivo. For example, deficiency states suggest that, for the most part, these two fat-soluble vitamins function independently. However, these classical nutritional studies do not exclude the possibility that each vitamin receptor may be important for the other's function in a ligand-independent fashion. Further studies to determine the validity of this mechanism and to determine whether it can be applied globally to other vitamin D- or vitamin A-mediated systems are required.

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